THE DEVELOPMENT AND EVALUATION OF A MULTIPLEX REAL TIME PCR ASSAY FOR THE DIAGNOSIS OF ACANTHAMOEBA AND HERPES SIMPLEX KERATITIS

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Introduction
The inflammation of the cornea caused by Acanthamoeba and herpes simplex virus is called Acanthamoeba keratitis (AK) and herpetic keratitis (HR) respectively. The most common symptoms of keratitis are eye redness, pain, blurred vision, photophobia, irritation and excess tearing.

Acanthamoeba is an opportunistic protozoan present in a variety of habitats as an active trophozoite or dormant cyst form. More cases of AK are reported in contact lens users (85-88%). HSV - 1 is a more common cause of ocular infection and can cause four types of keratitis. Accurate, fast diagnosis and treatment of AK and HK are essential to prevent the permanent damage of cornea and ultimately loss of sight.

Current diagnostic approaches are time-consuming, lack sensitivity, expensive and require further interventions.

Aims: Develop a Multiplex PCR assay that can detect AK and HK ensuring good quality of sample and authenticity of the process.

Methods
- Two sets of Acanthamoeba P+P used from previous journals.
- HSV P+P used from in-house Taqman PCR assay.
- A. castellani and AcroMetrix Multi- Analyte used as +ve controls.
- RNaseP used to determine corneal sample adequacy.
- DNA IC for the extraction process.
- The assays optimised and multiplexed together on LC PCR.
- 8 strains of Acanthamoeba cultured on Page’s agar plates.
- KOWA glassic slide used to count the cysts.
- NA extracted on Roche MagNaPure.
- Amplification was done on LightCycler PCR.
- Comparison between LC and Taqman with 26 HSV strong and weak positive samples.
- HSV clinical specimens spiked with Acanthamoeba +ve control.
- The reproducibility test performed on 5 serial dilution of Acanthamoeba and HSV positive controls run in replicates of 5 per run, performed on four days.
- 50 corneal scrapes and 60 eye swabs tested to determine SENSITIVITY and SPECIFICITY of the assay.

Results
- Acanthamoeba LOD = 1 copy of genome/ reaction
- HSV LOD = 1 genome in 10000 dilutions
- On Lightcycle, HSV Ct range = 18.74 – 37.10
- On ABI Taqman HSV Ct range = 18.86 – 44.24
- Efficiency of Multiplex PCR assay = 98% -100%
- Cell count = A. polyphaga max. clinical specimen mini
- Duplex PCR detects 1/1000000 dilution of A. strains
- Monoplex PCR assay detects 1/100000 dilution
- No detection of A. astronyx
- Reproducibility CoV of 0.822 and 0.602

Sensitivity = 100%, Specificity = 98% and 100%

<table>
<thead>
<tr>
<th>Assay</th>
<th>No of samples</th>
<th>PCR positive</th>
<th>Sensitivity (%) (95 % CI)</th>
<th>Specificity (%) (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthamoeba</td>
<td>50</td>
<td>3</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20% to 100%</td>
<td>88% to 100%</td>
</tr>
<tr>
<td>HSV</td>
<td>60</td>
<td>18</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>78% to 100%</td>
<td>90% to 100%</td>
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Discussion
During early stages Acanthamoeba keratitis usually represents with mimic herpetic epithelial keratitis. This can lead to either missed or delayed diagnosis of keratitis that can cause severe ocular damage. Therefore, the purpose of this study was to develop a multiplex PCR assay that can diagnose AK and HK simultaneously.

The corneal scraping sample is the preferred sample for reliable diagnosis of AK. This is because Acanthamoeba penetrates deep into cornea, therefore, superficial swabs or tears are often unsuitable for AK detection in advance stages of infection or if pre-treated with antibiotics.

The set of all primers and probes used in this assay run at a 60°C elonuation temperature; therefore, the assay can be loaded on a single PCR plate in one well to achieve high sensitivity and short turnaround time.

There are some limitations, such as, the new assay was unable to detect Acanthamoeba astronyx, a small number of positive Acanthamoeba were tested and the assay contains HSV probe 1 & 2 with same flourescence dye. Therefore, this project could be improved by using two separate fluorescence dyes for HSV probe 1 and 2.

Once the assay is put into routine use by Roche flow, it is expected that majority of the samples would be tested on the same day to get results within 24 hours.

This assay improves the turnaround time for keratitis diagnosis and reporting of the results to benefit patient management with use of appropriate and targeted antibiotic treatment.

Conclusions
- This Multiplex PCR assay provides better sensitivity and specificity to both Acanthamoeba and HSV also ensure the sample quality and extraction process.
- However, one culture positive Acanthamoeba strain (A. Astronyx) was not detected by this Multiplex PCR assay.
- Therefore, culturing of Acanthamoeba specimen cannot be discontinued, but will be augmented by PCR.
- This assay is a great success to provide fast and accurate diagnosis of Acanthamoeba and herpetic keratitis.

Acknowledgement
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References